Development of a Reverse Genetics System to Produce Live, Attenuated Infectious Salmon Anemia Virus (ISAV) Vaccine Candidates

Final Project Report

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Abstract

Infectious salmon anemia (ISA), induced by the viral causative agent infectious salmon anemia virus (ISAV), has had a large, negative economic impact on the salmon aquaculture industry in Maine, and has resulted in a considerable number of both direct and indirect job losses among communities near aquaculture operations. The goal of this project was to develop a reverse genetics system for ISAV to construct live viral particles from plasmid DNA molecules, with attenuated or reduced virulence that could induce a protective immune response in salmon and vaccinate against subsequent infection by ISAV in the environment. Although not complete, significant accomplishments towards this goal include: (i) Identification of errors in published ISAV genomic sequences. (ii) Development of procedures for high efficiency plasmid transfection into salmonid cells. (iii) Identification and cloning of a *Salmonid* Pol I promoter. (iv) Construction of dual-functional plasmids capable of promoting both mRNA and protein expression, as well as expression of negative strand viral, non-mRNA molecules in salmonid cells. (v) Demonstration of protein, mRNA, and viral RNA production in salmonid cells and incorporation of a plasmid-encoded viral RNA into extracellular viral particles.

Executive Summary

Although the final project goal – production of infectious, reverse-genetic engineered and attenuated ISAV virions – was not completed during the grant period, we believe the data from the experiments we were able to carry out give a strong indication of the ultimate success of the project. We have constructed and shown that dual functional plasmids, containing ISAV genomic segments under the control of mRNA (Pol II, poly-a tail) and non-mRNA (Pol I and Pol I terminator) gene control signals do produce both positive and negative strand RNAs, and that a viral genomic negative strand transcribed from a plasmid can be packaged into extracellular viral particles. Specific experimental accomplishments include:

(1) Identification of errors or incomplete sequences in the published GenBank ISAV sequences for seven of the eight ISAV genomic segments. After trying a variety of methods over the course of the grant period, we believe we have finally developed a procedure that will allow determination of the correct nucleotide sequence of both the 3' and 5' ends of all eight ISAV genomic segments.

(2) Development of procedures for high efficiency plasmid transfection into salmonid cells Transfection rates of up to 45% were obtained using a CHSE cell line with the "Transit LT" transfection reagent (Mirus, catalog # MR2300) giving the best results.

(3) Identification and cloning of a *Salmonid* Pol I promoter. Using published primers in conserved regions of the ribosomal rRNA locus (Reed. 2000) we cloned and then subcloned a fragment from *Salmo salar* from the Intergenic Spacer region and identified by sequence homology a putative Pol I promoter region.

(4) Construction and testing of a multifunctional expression plasmid backbone capable of promoting both mRNA and protein expression and negative strand viral (non-mRNA) molecules. We constructed a dual functional expression plasmid backbone for producing both positive strand mRNA and authentic, native negative strand viral RNA from the same ISAV segment insert. The CMV Pol II promoter and the SV40 late poly-adenylation signal are used to drive expression of mRNA. The cloned *Salmonid* Pol I promoter and a synthetic murine Pol I terminator

together drive expression of full-length uncapped, non-poly-A tailed viral RNA molecules. The remote cutting restriction enzyme *Bsm*BI and adapters containing *Bsm*BI recognition sites were used to allow cloning of all eight ISAV segment into the expression plasmid backbone in a manner that allows expression of native ISAV viral RNA segments with no extraneous or foreign bases at either end.

(5) Determination of the interferon production status of a variety of salmonid cell lines. Using detection of Mx protein, an interferon mediated anti-viral protein, we determined the interferon status of CHSE, ASK, and SHK cells. Mx protein was detected at low level in all cell lines.

(6) Construction and demonstration of functional protein expression from a plasmid transfected into salmonid cells. We constructed a plasmid with the GFP coding sequence inserted into our expression plasmid backbone, transfected this plasmid into a salmonid cell line and were able to detect both GFP mRNA and GFP protein.

(7) Demonstration of the functionality of the *Salmonid* Pol I promoter in salmonid cells. We used the same GFP plasmid transfected into salmonid cells described above to demonstrate, using strand-specific RT-PCR, the production of negative strand, anti-sense GFP RNA molecules demonstrating that our cloned Pol I promoter fragment was functional in salmonid cells.

(8) Construction of ISAV transcription plasmids for two of the eight viral segments. We cloned the complete ISAV segment 6 and segment 8 genomic sequences into our backbone expression plasmid and were able to demonstrate production of both mRNA and viral RNA from the segment 6 plasmid. Although when we sequenced these plasmid inserts both exhibited several single base changes from the published ISAV genomic sequences - probably PCR induced mutations - both inserts had the expected and correct terminal sequences, demonstrating the success of our cloning strategy. Using a segment 6 deletion expression plasmid, we were able to demonstrate packaging of negative strand, viral RNA produced from this plasmid into viral particles.

IV. Project Purpose

A. Description of problem addressed:

This project addressed specific priority A (Atlantic Salmon Aquaculture Development Considering the Endangered Species Status of Atlantic Salmon), listed in the NOAA/NMFS Notice for Solicitation for Applications published in the Federal Register Vol. 67, N0. 93, Section II. As presented in the solicitation notice in November of 2000 wild populations of Atlantic salmon in eight Maine rivers, from the lower Kennebec River north to the US – Canada border, were listed as endangered under the Endangered Species Act (ESA) (16 USC 1531-1544). Concern that interaction of farm-raised and wild salmon through competition, interbreeding, and disease might threaten wild salmon populations in the Gulf of Maine has placed the continuation of the industry in jeopardy.

The loss of Maine's aquaculture industry would be a serious blow to the state's economy. Maine's salmon aquaculture industry is the top producer of farm-raised salmon in the United States (36.7 million pounds in 2000) with a market gate value of $85 million (US) (World Aquaculture Society, 2001). The industry also provides over 2,500 jobs in economically depressed areas and generates $140 million in personal income. On a global level, as commercial exploitation of wild fisheries declines, and as world population grows to a projected 8 billion people by 2025 (Aquaculture Magazine Buyer's Guide, 2001), cultured finfish products will be in increasing demand and serve as an important protein source. The survival of Maine's cultured salmon industry will increasingly depend on eliminating or minimizing the potential for negative impacts on endangered wild Atlantic salmon. Two posited areas of great concern to regulatory agencies are the issue of disease resulting from possible environmental pathogen loading on migrating wild fish by cultured salmon populations, as well as the potential interaction of wild fish and disease carrier escapees from aquaculture operations. Both possibilities were referenced in the federal endangered species listing proposal for Atlantic salmon. Farmed fish and wild fish alike are at risk of being affected by many infectious agents and diseases. It is assumed that once infected with a pathogen, cultured fish stocked in high density at marine sites then pose a higher proportional pathogen transmission risk to wild stocks. Development of vaccines that could be used to protect cultured salmon populations against such infectious agents and diseases would greatly reduce or eliminate the risk of transmission of these pathogens to endangered wild Atlantic salmon. This proposal will focus directly on developing a technology for producing a vaccine against the most significant disease currently affecting the cultured salmon industry in Maine, Infectious Salmon Anemia.

Infectious salmon anemia (ISA), induced by the viral causative agent infectious salmon anemia virus (ISAV), is a disease of variable infectivity and mortality affecting Atlantic salmon (*Salmo salar*). First observed in salmon aquaculture operations in Norway in 1984 (Thorud et al. 1988), ISA was later described from Scotland (Rodger et al. 1998; Rowley et al. 1999), New Brunswick, Canada (Mullins et al. 1998; Blake et al. 1999; Bouchard et al. 1999; Lovely et al. 1999) and has recently appeared at numerous aquaculture sites in Cobscook Bay, Maine (Bouchard et al.

2001). The presence of this exotic pathogen and the disease experienced at Maine sites led to the massive destruction in late 2001 of more than 1 million Atlantic salmon raised at sites in Cobscook Bay, through a United States Department of Agriculture/Animal Plant and Health Inspection Service (USDA/APHIS)-sponsored ISA management program. To date, ISA has had a large, negative economic impact on the salmon aquaculture industry in Maine, and has resulted in a considerable number of both direct and indirect job losses among communities near aquaculture operations. Decreased revenue to the state of Maine through lower payroll and other taxes has also been realized, as well as a reduction in the amount of money paid collectively to the state of Maine by the salmon producing companies on a per-pound basis, which funds several state agency programs.

Complete characterization of the ISAV genome, together with recently developed "reverse genetics systems" to construct live viral particles from DNA plasmid molecules, now make it possible to produce live ISA virus particles with attenuated or reduced virulence that could induce a protective immune response in salmon and vaccinate against subsequent infection by ISAV in the environment.

B. Project Objectives:

The specific project goals and objectives were to apply techniques developed for other viral pathogens, such as the human orthomyxovirus, influenza, to the development of a reverse genetics system for ISAV as follows:

- Construct a complete set of plasmids containing the entire ISAV genome.
- Transfect the plasmids into salmonid cells and demonstrate functionality of the plasmids individually.
- Transfect the complete set of plasmids into salmonid cells and demonstrate the production of live, infectious ISAV particles.
- Demonstrate that mutations in a specific ISAV gene can attenuate the virulence of the reconstituted virions.

These live attenuated virions would be ideal vaccine candidates for testing in salmon in laboratory and field efficacy trials.

V. Project Approach

A. Detailed description of work performed

The project design consisted of two major experimental phases: Phase 1 experiments, primarily utilizing molecular and recombinant DNA techniques, focused on assembling a set of eight DNA plasmids that together contained the entire genome sequence of ISAV and encoded all the protein components necessary to completely reconstitute infectious virus particles when transfected into a salmonid cell line. Phase 2 experiments, primarily utilizing viral and cell culture techniques, focused on developing techniques for transfecting the plasmids constructed in phase 1 into salmonid cell lines and assaying mRNA and protein expression from the plasmids. Detailed descriptions of the project experimental accomplishments and findings are described in the Project Findings section, below.

B. Project Management

The project was jointly managed by John Wood, Ph.D. at Pisces Molecular LLC and William Keleher at Micro Technologies, Inc. as co-Principal Investigators. Pisces Molecular was the primary applicant for this project and was responsible for administering all funds awarded for this project and for all project reporting required by NMFS and the S-K Program Office. All molecular and plasmid construction experiments were carried out in the laboratories of Pisces Molecular, principally by John Wood and Janet Epp. All cell culture, virus and transfection experiments were carried out in the laboratories of Micro Technologies, principally by Cem Giray.

VI. Project Findings

A. Actual accomplishments and findings

Extensive work on a variety of segmented RNA viruses, particularly human influenza virus, has demonstrated that the terminal sequences of the segments have conserved sequences that are critical for both correct transcription and packaging into viral particles (Fodor, 2002). Therefore, having the correct ISAV segment end sequences was critical to the success our reverse genetics system - ensuring the correct functionality of segment expression plasmids and packaging of viral segments into virions. Although the complete sequence of all 8 ISAV segments was published and available in GenBank when we started this project, close scrutiny of the segment end sequences, as well as comparison with each other and with the human influenza segment end sequences, indicated that seven of the eight published ISAV segment sequences were incomplete or erroneous. Several of the published sequences were obtained from ISAV messenger RNAs – which are known to be shorter than their corresponding viral genomic segments – and were incorrectly described as "full length" sequences (Clouthier, et. al., 2002). One GenBank ISAV

segment sequence contained non-ISAV terminal sequence related to the cloning procedure – the sequence had perfect homology to the *E. coli* plasmid "multi-cloning site" common to nearly all cloning plasmids (Clouthier, et. al., 2002). Conversely, two of the published ISAV segment sequences, for segment 6 and segment 8, did have what appeared to be correct and conserved terminal sequences (Sandvik, et. al., 2000). However, for segment 8, although both termini and the protein coding sequence are known (Sandvik, et. al., 2000; Clothier, et. al. 2002); the sequence of the untranslated region between the stop codon of the coding sequence and the 5' terminus (of the antisense, viral strand) had not been determined.

Determining the correct terminal sequences of the ISAV genomic segments has been a source of great frustration throughout this project. Although not part of the original proposal, solving this technical problem and determining the ends of ISAV segments numbers1 through 5 and 7, was essential to the successful completion of this project. Using a modification of the RACE technique (Rapid Amplification of 5'cDNA Ends), using a commercially available RACE kit from Clontech, we were successful in determining the 5' end of ISAV segment 1 (Figure 1); however this technique did not work for two additional ISAV segments (#2 and #3) that we tried. In both cases

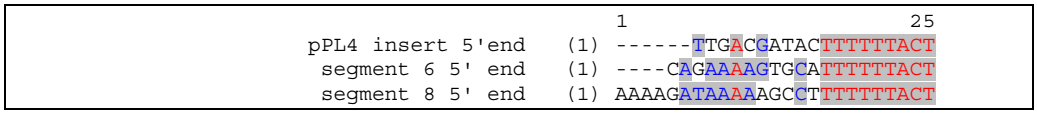

Figure 1: ISAV Segment 1, 5' RACE reaction clone. The top sequence is that determined by RACE amplification, cloning and sequencing of the 5' end of ISAV segment 1. The bottom two sequences are the 5' ends of ISAV Segments 6 and 8 determined by Sandvik, et al. (Sandvik, 2000). Note the conserved sequence of the 9 terminal bases of all three segments – which base pair with a complementary sequence at the 3' end of the segments, forming each genomic RNA segment into a hairpin loop believed to be essential for correct/efficient packaging of the RNA segments into viral particles.

although the RACE reactions appeared to work well (as shown by kit controls and the appearance of the expected size ISAV fragments) and we correctly amplified the intended ISAV segments, we did not reach the 5' end of either of these two segments, ash shown in Figure 2. Scouring the available scientific literature, much discussion with

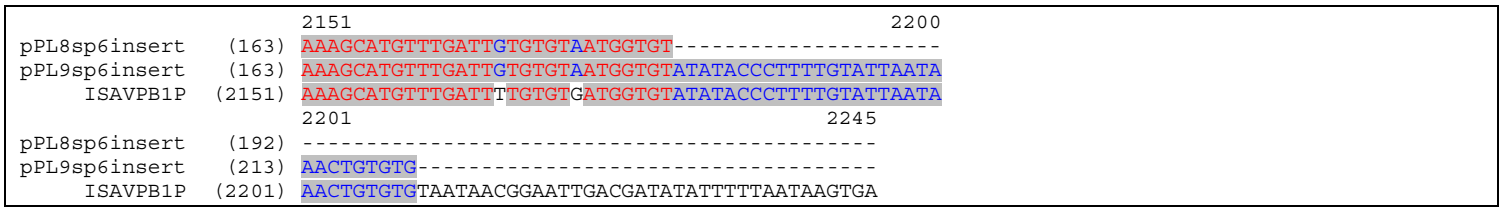

Figure 2: ISAV Segment 2, 5' RACE ends. The top two sequences are those determined by RACE amplification, cloning and sequencing of the 5' end of ISAV segment 2, from two independent clones. The bottom sequence is Genbank submission (AJ002475) for ISAV Segment 2 (Krossoy, 1999).

Clontech technical support personnel, and email correspondence with the authors that were able to determine the ends of two ISAV segments (#6 and #8; Sandvik, 2000) all indicated that determining the 5' end of viral RNA segments was a very difficult problem. All RACE kits, including the Clontech kit – and even the ubiquitous Cold Spring Harbor Molecular Cloning Manual (Sambrook, 2001) - suggest that the RNA polymerases used in RACE reactions have great difficulty copying all the way to the end of RNA molecules with high degrees of secondary structure. Unfortunately, it is precisely because the ISAV segment ends are likely have significant secondary structure – essential for correct packaging of the genomic segments into assembling viral particles (Sandvik, 2000) – that we needed to determine the sequence of these ends. At its most basic level, the problem seemed to be that the complete, full-length-to-the-end RACE reaction was a low probability event, and yet there was no easy way to screen or select for these low frequency occurrences.

After exhaustive efforts with RACE reactions, we tried an alternative technique used for determining the ends of influenza viral segments (Symkowiak, et. al., 2003). Rather than relying on amplifying and sequencing the ends of fragments, with all the attendant problems of segment "ends", we circularized the viral segments, eliminating the ends, as shown in Figure 3a (note Figure 3 is large and is therefore appended at the end of this report rather than being integrated with the text). We used T4 RNA ligase (NEB catalog # M0202S) to ligate the two ends of a viral segment together to form a single stranded RNA circle (Figure 3a), then used an appropriately designed primer to reverse transcribe into DNA a portion of the circular RNA molecule spanning the ligation junction (figure 3b), then PCR amplified a similar junction-spanning fragment (Figure 3c). Finally, we cloned the PCR amplicon into a T/A plasmid cloning vector and transformed into *E coli*. (Figure 3d). Bacterial clones with inserts (white colonies) were screened for inserts of the predicted size by PCR using additional segment primers (Figure 3e). Plasmids were prepped from a number of clones with the predicted size insert and the insert DNA sequenced. We started with segments 6 and 8,

where the complete terminal sequences were already known, as a control to test whether the procedure was working. Running an aliquot of the PCR reaction (Figure 3c) on a gel did show a diffuse band of the appropriate size, suggesting that RNA ligation, reverse transcription, and PCR amplification steps were all working. The diffuseness of the PCR bands suggested sequence heterogeneity in the amplicons; therefore rather than attempting to directly DNA sequence from the PCR amplicons, we cloned the PCR reactions into a T/A cloning vector (Qiagen pDrive, catalog # 231224) transformed *E. coli*, and selected white bacterial colonies containing plasmids with inserts. When we screened the plasmid clones, using PCR primers complimentary to plasmid sequences, we did find a variety of insert sizes: The expected size of the segment 6 insert was 430 bp, the 120 clones we screened contained inserts from approximately 300 to 600 bp in size. Similarly for the segment 8 insert: we expected an insert of 334 bp; in 150 clones we observed inserts from 300 to 1000 bp in size. We picked several plasmid clones with the expected size insert for both segment 6 and segment 8 and DNA sequenced them. The resultant sequences and their alignment with each other and with the predicted segment 6 and segment 8 terminal sequences are shown in Figure 4.

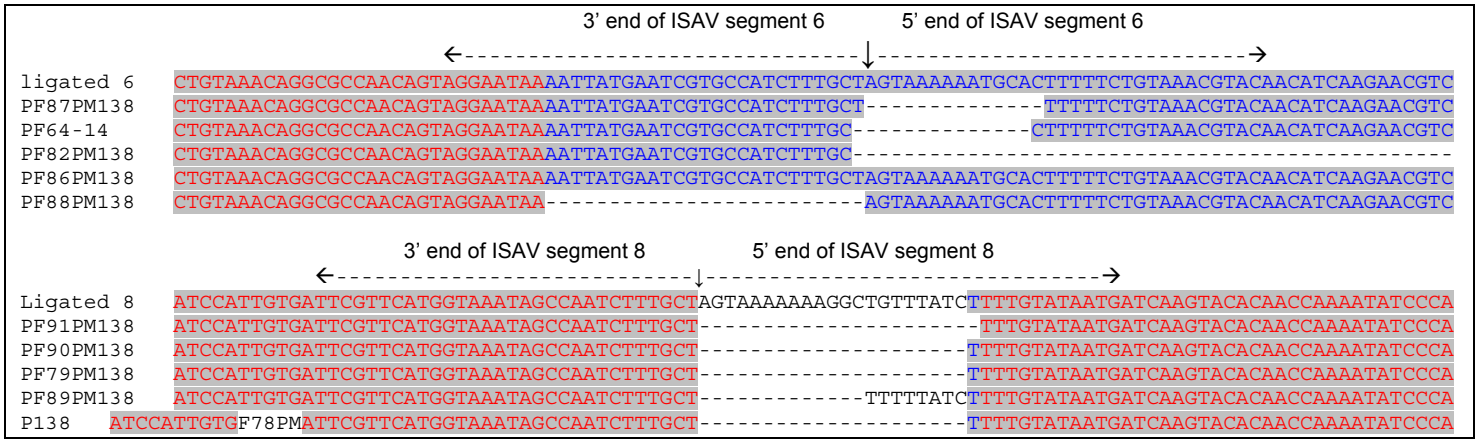

Figure 4: DNA sequences surrounding the ligation junction of cloned, circularly ligated and amplified ISAV segment 6 and segment 8 termini. The predicted ligated ISAV 6 and ligated ISAV 8 sequences are constructed from an assembly of segment 6 and segment 8 sequence termini, respectively (Sandvik, et. al., 2000, Clothier, et. al. 2002, and J. Epp, unpublished). Down arrows indicate the ligation junction. Shaded positions indicate sequence identity with the predicted ligated sequence; dashes indicate deletions relative to the predicted sequence

In nearly all cases, the 3' sequences we obtained match the Sandvik, et. al. sequences exactly. However, nearly all the 5' sequences all contain one or more deletions, ranging from a minimum size of 6 nucleotides to as large as 99 bases. One plasmid clone did give a sequence for the 5' and 3' termini of segment 6 identical to that of Sandvik (PF86PM138 in Figure 4). All of the eight segment 8 plasmid clones we sequenced were shorter than predicted and did not contain DNA sequences homologous to the 5' terminus of segment 8. Therefore we were successful in cloning and determining the 5' terminal sequence for ISAV segment 6; however this procedure appears to give a low yield of full length molecules, similar to the results observed using this technique to determine the ends of influenza virus segments (Szymkowiak, et. al., 2003). As illustrated in Figure 4, the deletions observed in the clones we sequenced, both in segment 6 and segment 8 clones, exhibited a striking asymmetry in their location – in all cases except one the deletion was confined to sequences at the 5' terminus of the native viral segment. We interpreted this asymmetry to indicate that the 5' end of the native viral segment RNAs is likely blocked and/or incapable of ligating - only viral segment molecules that have been broken or deleted at the 5' end are able to ligate. RNA ligase, like DNA ligases, requires a 5' PO_4 group and a 3' OH group; without both of these chemical groups ligation does not occur. There is no published information on the chemical nature of ISAV segment ends; however, the literature suggests less than complete agreement for the exact chemical nature of the 5' terminal end of influenza segments – either 5'-PO_4 (monophosphate) (Szymkowiak, et. al, 2003) or 5'-$PPPO_4$ (triphosphate) (Young & Content, 1971). Our data suggest that ISAV segments do not have terminal 5' monophosphates. We tested the simplest possibility – that circular ligation was infrequent because the 5' segment ends had no phosphates at all (5'-OH) - using T4 polynucleotide kinase (NEB catalog #M0201S) to add a 5'monophosphate to the 5' terminal nucleotide of the viral segment termini segment before ligating (prior to step 1(a) above). These experiments were not successful in increasing the efficiency of RNA ligation: incubation of the RNA preparation with kinase prior to RNA ligation and PCR resulted in weaker and more heterogeneous in size amplification products – suggesting a decrease the efficiency of PCR amplification after RNA ligation, rather than an increase. Although there are published procedures for using calf alkaline phosphatase to remove 5' triphosphates, then using kinase to add back a monophosphate (Skehel & Hay, 1978), these are cumbersome protocols as the two enzymes require different

reaction buffers, requiring loss-causing purifications steps between the different enzymatic steps. A simpler alternative took advantage of the different viral RNAs produced during viral replication. When ISAV, or other orthomyxoviruses, infect a cell, the viral RNA polymerase synthesizes a variety of RNA molecules (Figure 5a). The first viral RNAs detected are mRNA molecules, with 5' caps and poly-A tails, from each of the viral segments. Later, in a poorly understood process Mikulasova, et. al, 2000), the viral polymerase begins to synthesize full-length, positive strand cRNA molecules; still later these cRNAs serve as template for synthesis of full length, negative strand viral RNA molecules (vRNA). The ISAV RNA preps we used were from infected kidney cells; therefore all three forms of viral RNA should be present in the RNA preps. In designing the initial RNA circular ligation and amplification procedure diagrammed above in figure 4, we expressly targeted reverse transcription and PCR amplification of only the vRNA strand – because the added RT primer was sequence <u>complimentary</u> only to the negative strand vRNA (and <u>homologous</u> in sequence to the positive cRNA strand; see Figure 5b). Targeting the cRNA instead only requires using a primer complimentary to the cRNA sequence (and homologous to the vRNA sequence; Figure 5c). Since the vRNA targeting assay gave a complete 3' terminal sequence but incomplete or deleted 5' terminal sequences, then a cRNA targeting assay should give similar results – but the <u>3' terminal cRNA</u> sequence is the reverse compliment of the desired but elusive <u>5' terminal vRNA</u> sequence. Therefore by ligating our total RNA prep, containing both vRNA and cRNA (as well as mRNA), then reverse transcribing and PCR amplifying separately with both a vRNA RT primer and a cRNA RT primer, then cloning sequencing, and aligning molecules of both populations we should obtain the complete sequence of both the 3' and 5' viral termini (Figure 5c). Additionally, the intersection of the respective 5' vRNA and 3' cRNA deletion sets should locate the junction where the original 5' and 3' ends ligated and allow demarcation of the authentic 5' and 3' viral segment ends. These experiments are still currently in progress.

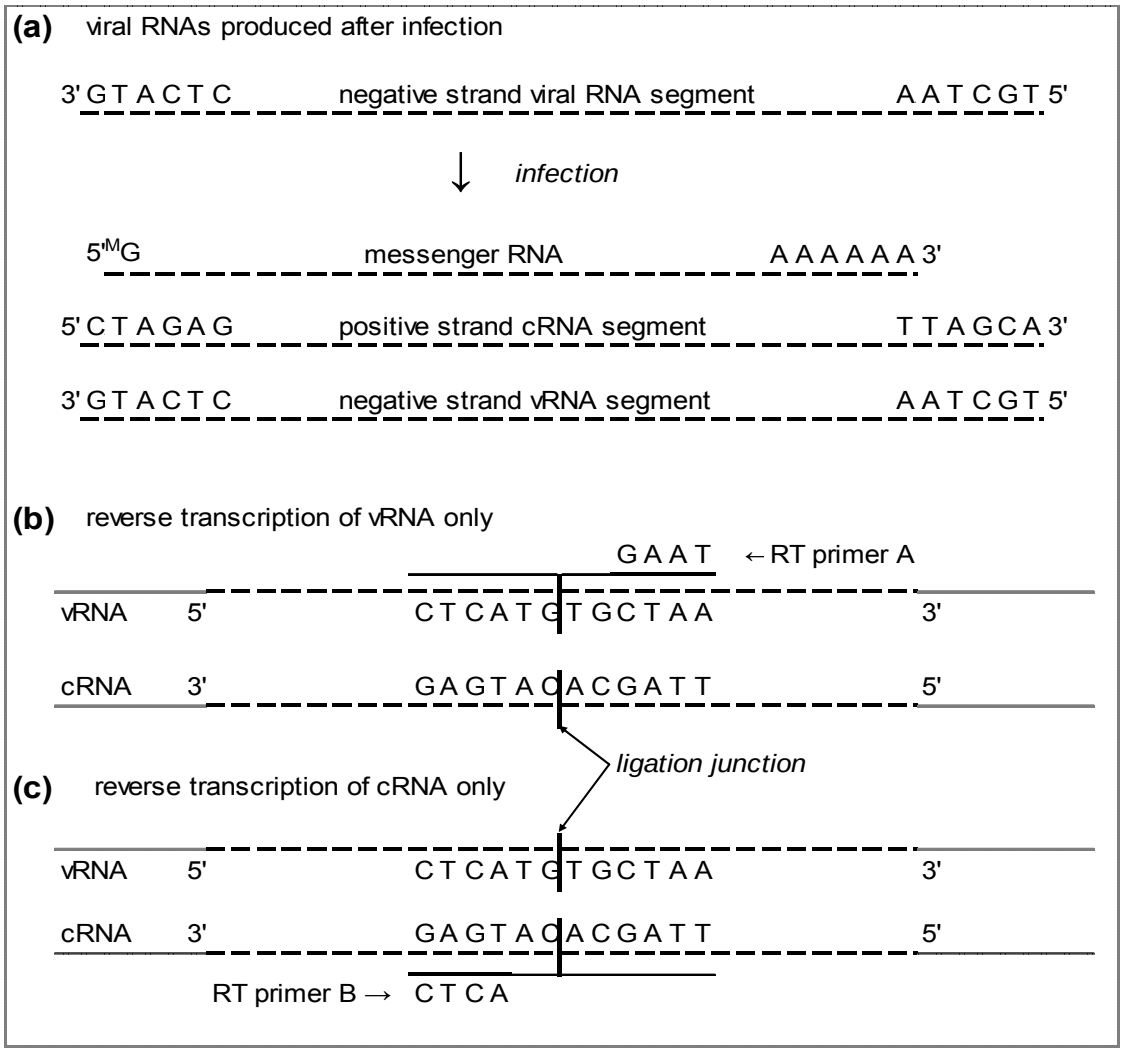

Figure 5: Viral RNA molecules and targeting vRNA or cRNA

Although the difficulties with determining ISAV segment ends prevented finishing the ultimate project goal of assembling a complete, reverse-genetically engineered and attenuated ISAV, we did have a number of other significant accomplishments.

We were able to obtain high efficiency transfection of salmonid cells. Three cell lines were selected for the first round of transfection optimization strategies; CHSE-214, SHK, and ASK cells. Six different transfection reagents were researched and samples requested from vendors. A preliminary experiment was designed to determine

the toxicity effects and levels of the transfection reagents alone on the three selected fish cell lines by evaluating a range of levels above and below the manufacturer's recommended levels. The preliminary experiment aided in establishing appropriate concentration ranges and times of exposure for the selected transfection reagents as most transfection reagents are designed for mammalian cells grown at temperatures much higher than fish cell lines. Four transfection reagents were selected for the transfection experiment using the Green Fluorescent Protein (GFP) plasmid obtained from project consultant Michael Bouchard. Transfection reagent concentration, time of exposure, and DNA concentration were all compared using the three cell lines in order to, first, determine if transfection of the three cells lines was possible and, second, optimize the transfection efficiencies. The results of these experiments did demonstrate successful transfection of the CHSE cells with three of the transfection reagents evaluated. Typical to the slow growth rates of fish cells in culture, observable transfected CHSE cells increased over six days. Transfection rates of up to 45% were obtained using the CHSE cell line, with Transit LT giving the best results. ASK and SHK cell lines did not show adequate transfection for our purposes.

Another task of the project was to determine or find a salmonid cell line that did not produce interferon. An interferon negative (IFN-) line would be used to grow the attenuated (NS-1 deletion) reverse-engineered virions, as well as to compare the virulence of both the wild type (NS-1+) and attenuated (NS-1 deletion) virions, on interferon positive and interferon negative cell lines. The ability of CHSE, ASK and SHK cell lines to produce interferon was tested using the detection of Mx protein, an interferon mediated anti-viral protein. Mx protein was detected at low levels in all cell lines. A dose response was observed in ASK, SHK and one strain of CHSE cells when treated or transfected with Poly I:C, a known inducer of Mx protein and in turn, interferon activity. One strain of CHSE cells did not show induction of Mx protein when treated with Poly I:C. Experiments comparing Mx protein production in cells transfected with Poly I:C in the presence and absence of ISAV to determine the effect of ISAV on interferon production in the three cell lines indicated that ISAV had a smaller effect on Mx expression than poly I:C in all three cell lines.

Another project goal was to construct a plasmid containing the coding sequence of the Green Fluorescent Protein (GFP) under the control of gene expression signals that would function correctly in salmonid cells, and demonstrate correct and functional protein expression by detecting the green fluorescence of active GFP in cells transfected with the constructed plasmid. However, the easy success detecting green fluorescence in transfectants made with a generic GFP plasmid in salmonid cells lines rendered this task unnecessary. The gene control signal for GFP expression in the plasmid we used is the Cytomegalovirus immediate early promoter-enhancer (CMV-IE) region (Aruffo and Seed, 1987), which appears to function well, controlling/promoting heterologous protein expression in salmonid cells lines. Therefore, we decided to use this CMV-IE promoter in constructing our ISAV protein expression plasmids.

Although not as dramatically condensed as the previous experimental task, we were also able to simplify construction of the ISAV segment transcription plasmids another project goal. Nearly all RNA transcription control signals – including those in the CMV-IE promoter region described above – are read and then transcribed by RNA polymerase II to produce messenger RNA molecules with a 5' cap and a 3' poly-A tail. Since the transcription plasmids we were constructing need to allow synthesis of "bare" RNA molecules, without the 5' cap or 3'poly-a tails and identical to native ISAV RNA genomic segments packaged in virions, the transcription plasmids required a non-RNA polymerase II promoter/transcription control signal. RNA Polymerase I (Pol-I) and associated Pol-I promoter/transcription signals are ideal for this purpose. RNA Polymerase I and Pol-I transcription signals are used in eukaryotic cells for transcribing the non-5'-capped and non-3'-poly-A tailed precursor RNA molecule from which the 5.8S, 18S, and 23S structural ribosomal RNA molecules are cut. Although Pol-I promoter sequences have been identified from a wide variety of organisms none have been identified in any *Salmonid* species – and nearly all Pol-I promoters identified to date function only in cells of their native species (reviewed in Paule, 1998). Therefore, we identified and cloned a *Salmonid* Pol-I promoter sequence for constructing our transcription plasmids. Using PCR primers in conserved regions of the ribosomal RNA repeated locus (Reed, 2000), we cloned a 3.7 kb fragment of *Salmo salar* genomic DNA encompassing the rRNA locus, from the Intergenic Spacer region through the 5' end of the 18S rRNA gene. This fragment was subcloned in pUC19 into four separate smaller KpnI fragments of size 1300, 1200 bp 1100 bp, and 190 bp. Using restriction fragment analysis with restriction sites relatively conserved in this region across *Salmonid* species (brook trout, brown trout, lake trout, and North Atlantic salmon; Reed, 2000) and mapping of our primer locations, we narrowed down the probable location of the Pol-I promoter sequence to within the 1100 bp fragment. We sequenced the entire 1100 bp fragment, and by searching this sequence using the conserved sequence motif of Pol-I promoters (Paule, 1998, p 41), identified several potential Pol-I promoter sequences, including one very strong candidate region.

We constructed and tested a multifunctional expression plasmid backbone capable of promoting both mRNA and proteins expression and negative stand viral (non-mRNA) molecules. In our original proposal we had separated the tasks of demonstrating RNA transcription and viral protein expression, and further divided these from

construction and testing of the final ISAV viral segment transcription plasmids. As discussed in an interim progress report (February 2005), we decided that rather than devoting the effort necessary to make a separate and limited use transcription "test plasmid", the more useful ISAV segment transcription plasmids could also be used to demonstrate that we were getting RNA transcription from our plasmids. As described in the same progress report, we also evaluated two alternate approaches to getting both viral RNA segments and viral proteins expressed. For human influenza virus two alternative reverse genetics systems have been developed. One system, developed by Palese and coworkers, used 8 viral segment expression plasmids and 4 additional viral protein expression plasmids (Schickli, 2001) The other influenza reverse genetics system, developed by Neumann, Hoffmann and coworkers used 8 plasmids that were dual-functional producing both viral RNA segment plus viral protein expression (Hoffmann, 2000). Data from Hoffmann suggested that the efficiency of ISAV virion assembly would be higher when all viral proteins were expressed (from 8 dual functional plasmids), than if only the viral RNA polymerase subunits and NP (nucleoprotein) proteins were expressed initially (from 8 viral segment transcription plasmids and 4 viral protein expression plasmids). Therefore, we revised our original proposed approach (8 transcription plus 4 protein expression plasmids, *a la* Palese), and instead switched to constructing 8 dual-functional ISAV plasmids (*a la* Hoffmann).

The dual functional plasmids required gene control signals at both ends of the inserted ISAV segment, as shown in Figure 6 below. To make positive strand ("sense") mRNA molecules, an RNA polymerase II (Pol II) promoter signal is required upstream of the viral segment and a "poly-A tail" transcription termination signal downstream of the segment. Conversely, making negative strand ("antisense") viral RNA molecules requires an RNA polymerase I (Pol I) promoter signal "downstream" of the viral segment, and a Pol I terminator signal upstream of the segment. We assembled these gene control signals from a variety of sources. The basic *E. coli* plasmid (pUC19 origin and ampicillin resistance gene), together with the CMV Pol II promoter and SV40 poly-A tail signal were from plasmid pCI from Clontech. The CMV Pol II promoter is the immediate early promoter-enhancer region from Cytomegalovirus (CMV^{IE}) (Aruffo and Seed, 1987), which we previously demonstrated functions well in our salmonid cell lines to promote expression of a heterologous foreign protein, Green Fluorescent Protein (GFP). The poly-A mRNA transcription termination signal is the simian virus 40 (SV40) late poly-adenylation signal (Proudfoot, 1991). The Pol I promoter was the *Salmonid* Pol I promoter we had identified and cloned. Pol I terminator signals, in contrast to Pol I promoters, appear to be non-species specific and widely functional in heterologous cells, therefore we constructed from synthetic oligonucleotides a sequence to match that of the murine Pol I terminator (Zobel, 1993).

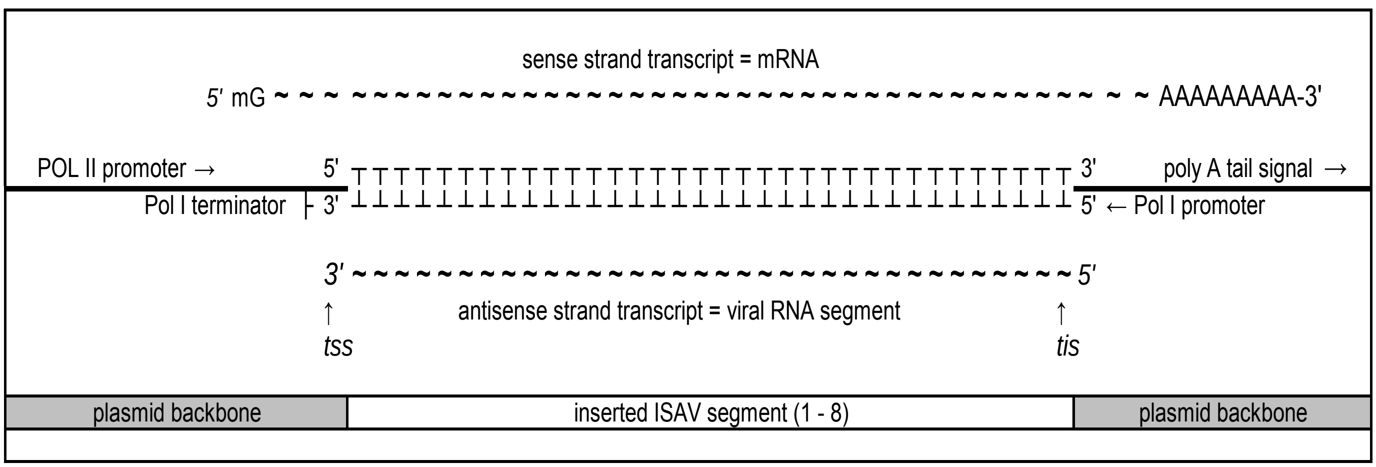

Figure 6: Arrangement of Dual Functional Gene Control Signals

Not surprisingly for a plasmid as complex as this, physically assembling the plasmid with all of the gene control signals in the correct orientation and spacing required a number of manipulations, as outlined in Table 1. All PCR reactions were carried out with high-fidelity DNA polymerase, (DeepVent from New England Biolabs or BD Advantage 2 Polymerase from Clontech) to minimize the risk of PCR-induced sequence changes/mutations in the amplified fragments. The first step in the plasmid assembly was to remove two superfluous BsmBI sites in the backbone PCI plasmid, so that BsmBI sites could be used later in the ISAV segment insertion point. The next step was to insert the synthetic murine Pol I terminator between the EcoRI and KpnI sites in the multi-cloning site of the plasmid. This was done by designing and synthesizing oligonucleotides to match the murine Pol I terminator sequence, annealing the two strands, cutting the resulting double stranded terminator fragment and the plasmid with EcoRI and KpnI, then ligating these two fragments together. In a similar fashion, the *Salmonid* Pol I promoter fragment was inserted into the KpnI and XbaI sites in the plasmid in the final construction step.

Two BmsBI restriction sites were engineered into the plasmid as the insertion sites for the ISAV segments. BsmBI is a "remote cutting" restriction enzyme with the strand cut sites outside of the restriction enzyme recognition site, and at indeterminate bases ("N", see box). By orienting the two recognition sites in opposite directions (with the cut sites "away" from each other, Figure 8a), when the plasmid is cut with BsmBI, both BsmBI recognition sites are removed and the plasmid is left with overhanging ends. Because these overhanging ends are not part of the BsmBI recognition site and are indeterminate, they can be engineered as any particular sequence desired (Figure 8b, page 10). Amplifying any of the ISAV genomic segments with primers that also contain BsmBI sites (Figure 8c) produces fragments that when cut with BsmBI will have overhanging ends compatible with the overhanging ends of pPL26 (Figure 8d). By carefully engineering the base sequence between the BsmBI recognition sites and cut sites in both the plasmid and in the ISAV segment amplification primers, we could insert the ISAV segments into the plasmid exactly at the Pol I promoter transcription initiation site (***tis***) and murine terminator transcription stop site (***tss***) with no extraneous or foreign bases at all (Figure 8e). Therefore transcription from the Pol I promoter of ISAV viral segments inserted into the plasmid in this manner will produce ISAV RNA molecules absolutely identical to native viral RNA segments. Work from both of the influenza reverse genetic systems (Schickli, 2001; Hoffmann, 2000) suggests that authentic viral segment ends are likely crucial for both proper virion assembly (viral segment packaging) and virus gene expression. Ensuring authentic ISAV segment ends also had the fortuitous effect of obviating potential concerns about foreign sequences in the final, live attenuated vaccine virus. The correct nucleotide sequence around the BsmBI insertion sites in pPL26 was confirmed by DNA sequencing.

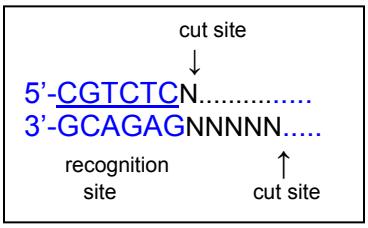

Figure 7 BsmBI remote cutting

Step	Purpose	Action
A	Remove 2 BsmBI sites from plasmid backbone to allow use of new, engineered BsmBI sites in ISAV segment insertion	PCR amplify pCI *E. coli* plasmid (Clontech) with outward facing primers on either side of BsmBI sites
		Purify PCR amplicon, ligate & transform *E. coli*
		Screen transformants for correct plasmid (shortened, no BsmBI sites)
B	Insert murine Pol I terminator between EcoRI and KpnI sites	Design & synthesize synthetic oligonucleotides to match murine terminator sequence with BsmBI site at transcription stop site (***tss***) and with RI & KpnI ends
		Anneal oligos to make double stranded terminator fragment
		Cut dsDNA terminator w/ EcoRI & KpnI
		Cut plasmid from step A w/ EcoRI & KpnI
		Ligate plasmid & terminator, transform *E. coli*
		Screen transformants for correct plasmid (correct fragment size for murine terminator insertion)
C	Insert *Salmonid* Pol I promoter between KpnI & XbaI sites	Design & synthesize primers to amplify Pol I fragment with KpnI & XbaI ends, internal BsmBI site, correct orientation & spacing relative to Pol I promoter transcription initiation site (***tis***)
		PCR amplify Pol I promoter fragment
		Purify amplicon, cut w/ XbaI & KpnI
		Cut plasmid from step B w/ XbaI & KpnI
		Ligate plasmid & Pol I fragment, transform *E. coli*
		Screen transformants for correct plasmid (correct fragment size for Pol I fragment insertion
D	Check final plasmid construct – pPL26 - by sequencing	Design & synthesize sequencing primers in plasmid backbone, external to engineered region (CMV Pol II, BsmBI sites, Pol I, poly-A tail)
		Use primers in dideoxy sequencing reaction to generate & fluorescently label ddX products
		Run sequencing reaction on a DNA sequencer
		Check sequencer readout against expected sequence

Table 1: Dual Functional Plasmid Construction Steps

a. pPL26 with BsmBI sites

```
                                        BsmBI
ACCTCCGAAGTTGGGGGGGAGGAGACGGTACCGTCTCGATATAGCTTTTCAAAA
TGGAGGCTTCAACCCCCCCTCCTCTGCCATGGCAGAGCTATATCGAAAAGTTTT
                        BsmBI
```

b. pPL26 digested with BsmBI

```
ACCTCCGAAGTTGGGG                          ATATAGCTTTTCAAAA
TGGAGGCTTCAACCCCCCCT                          TCGAAAAGTTTT
```

c. ISAV segment amplified with primers w/ BsmBI primers

```
ACGTCTCCGGGACG------------//--------------ACTATATCGAGACGT
TGCAGAGGCCCTGC------------//--------------TGATATAGCTCTGCA
          ↑~~~~~~~~~~ISAV segment X~~~~~~~~~~↑
```

d. Amplified ISAV segment cut w/ BsmBI (pPL26 compatible ends)

```
      GGGACG------------//--------------ACT
          GC------------//--------------TGATATA
        ↑~~~~~~~~~~ISAV segment X~~~~~~~~~~↑
```

e. pPL26 with ISAV segment inserted

```
ACCTCCGAAGTTGGGGGGGAGC-----//------ACTATATAGCTTTTCAAAA
TGGAGGCTTCAACCCCCCCTCC-----//------TCATATATCGAAAAGTTTT
                    ↑~~~~ ISAV segment~~~~~↑<------Pol I promoter
                   tss                     tis
```

Figure 8: Insertion of ISAV Segments into Backbone Plasmid pPL26

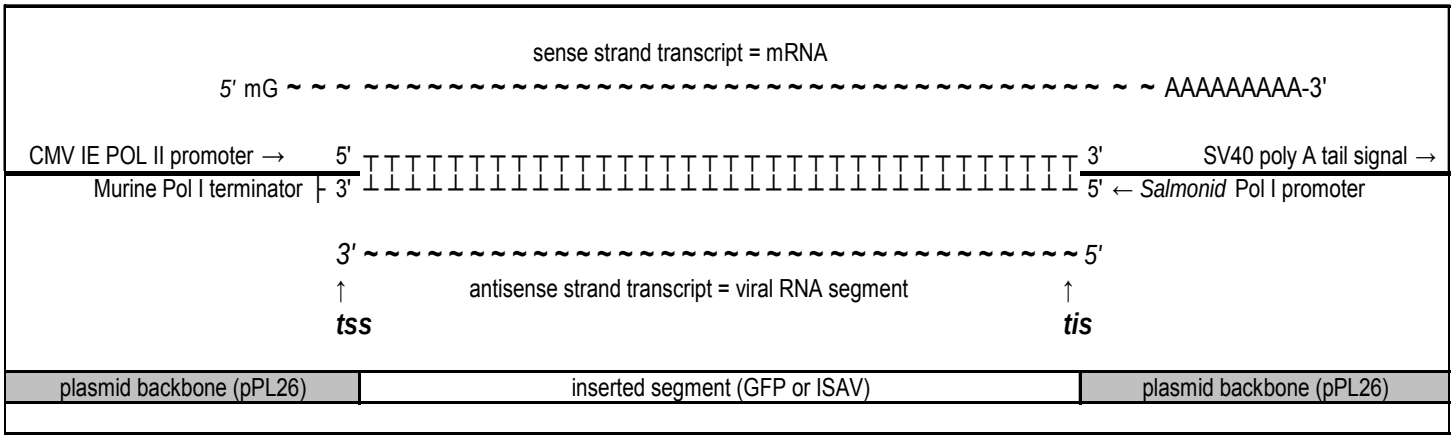

Figure 9: Arrangement of Dual Functional Gene Control Signals in pPL26

We constructed plasmids with four different DNA sequences inserted into pPL26 as shown in Table 2. Plasmid pPL28, containing the GFP (Green Fluorescent Protein) coding sequence was transfected into CHSE cells (both the ATCC type CHSE-214 cell line (CRL-1681) and an independent CHSE line maintained at Micro Technologies). Green fluorescent transfectants were observed with both cells lines (average transfection frequency =

30-40%), but not with mock transfected cells, demonstrating that pPL28 transfected cells were producing positive strand ("sense") RNA from the CMV promoter, and that this RNA was functional in CHSE cells as mRNA for expression of GFP protein. To test for production of negative strand ("antisense") RNA from the *Salmonid* Pol I promoter, we extracted total RNA from cell + supernatant aliquots of both transfected lines and the mock transfected cells. Residual plasmid DNA, which would confound the analysis of the RNA produced, was eliminated from the RNA preps by treating them with DNase (RNase free DNase, Qiagen, catalog # 79254). Subsequent testing of the DNase treated RNAs by PCR, using primers homologous to sequences in the plasmid pPL26 backbone (PM172 & PM174, outside the GFP coding sequence, Figure 10), gave no amplification, proving that the RNA preps were free of plasmid DNA. We then used a strand-specific two-step reverse transcription PCR protocol to test for the presence of negative strand GFP RNA. As shown in Figure 3, by using only a single primer in the reverse transcription step, we were able to distinguish negative strand GFP RNA from positive strand GFP RNA (which must be present in the transfectant RNA preps, based on their production of GFP protein). Using primer PM179, which can hybridize only to negative strand GFP RNA, and r*Tth* polymerase (Applied Biosystems, catalog # N8080098) in a reverse transcription-only (RT) step (1x 30 min @ 60°, no melt + anneal PCR cycles) would produce positive-strand GFP cDNA only if negative strand GFP RNA was present in the RNA preps. The RT reactions were cleaned up over a PCR clean-up column (Qiagen, catalog # 28104) to remove the r*Tth* enzyme and single-strand RNAs, prior to further, conventional, PCR amplification (r*Tth* because it could amplify positive strand GFP RNA during the PCR cycling, and single-strand RNA because even standard *Taq* DNA polymerase has been demonstrated to have a low

plasmid #	inserted sequence
pPL28	GFP coding sequence
pPL32	ISAV segment 8
pPL33	ISAV segment 6
pPL34	ISAV segment 6Δ (*Bgl* II del)

Table 2 Expression & transcription plasmids

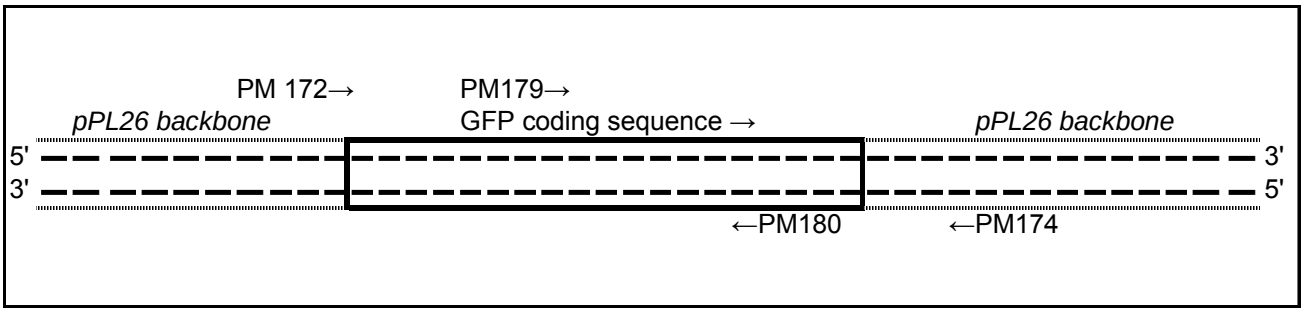

Figure 10 Primers used in GFP plasmid experiments. Primers above the plasmid sequence are "sense" rimers; i.e. they have the same base sequence as the positive sense strand of the plasmid and hybridize with the complimentary sequence on the negative strand of the plasmid. Likewise, primers below the plasmid sequence are "antisense" primers; i.e. they have the same base sequence as the negative, antisense strand of the plasmid and hybridize with the complimentary sequence on the positive strand of the plasmid

level of reverse transcriptase activity (Chandler, 1998). The PCR reactions were carried out with primers PM179 and PM180 (Figure 11) and hot-start Taq (AmpliTaq Gold, Applied Biosystems, catalog # N8080247). The results were that both transfectant RNA preps gave amplicons of the expected size; the RNA prep from mock-transfected produced no amplicons. This indicated that pPL28 transfected cells were producing negative strand GFP RNA from the *Salmonid* Pol I promoter.

We next carried out a set of experiments to determine the exact transcription initiation start point (*tis*) for this negative strand GFP RNA. Although we had tentatively identified the *tis* in our cloned *Salmonid* Pol I promoter fragment, based on sequence homology to Pol I promoter *tis* sites in other species (Paule, 1998), proof that this *tis* site was correct is crucial to ensuring that ISAV genomic segments inserted into pPL26 will be transcribed into negative strand RNAs with the correct, authentic ISAV terminal 5' sequence – which in turn, literature reports (Sandvik, 2000) suggest is necessary for packaging of viral RNAs into viral particles. Initially, we tried to use a RACE (Random Amplification of cDNA Ends) protocol to determine the 5' end of the negative strand GFP RNA. However, similar to our RACE experiments to identify segment ends, these experiments gave inconsistent and implausible results – at face value, suggesting that transcription of the negative strand GFP RNA started ~900 bp upstream of the putative *tis* site, in the SV40 polyadenylation signal sequence of pPL26, completely outside of our cloned *Salmonid* Pol I promoter fragment. As an alternative to RACE, we used two step RT-PCR with "sense" primer PM179 present during the RT step and two different "antisense" primers present during the PCR step, similar to the experiments described above and in Figure 11. As shown in Figure 12, using DNase-treated RNA preps from pPL28 transfectants in an RT step with only PM179 present would result in synthesis of a cDNA molecule extending only up to the 5' end of the

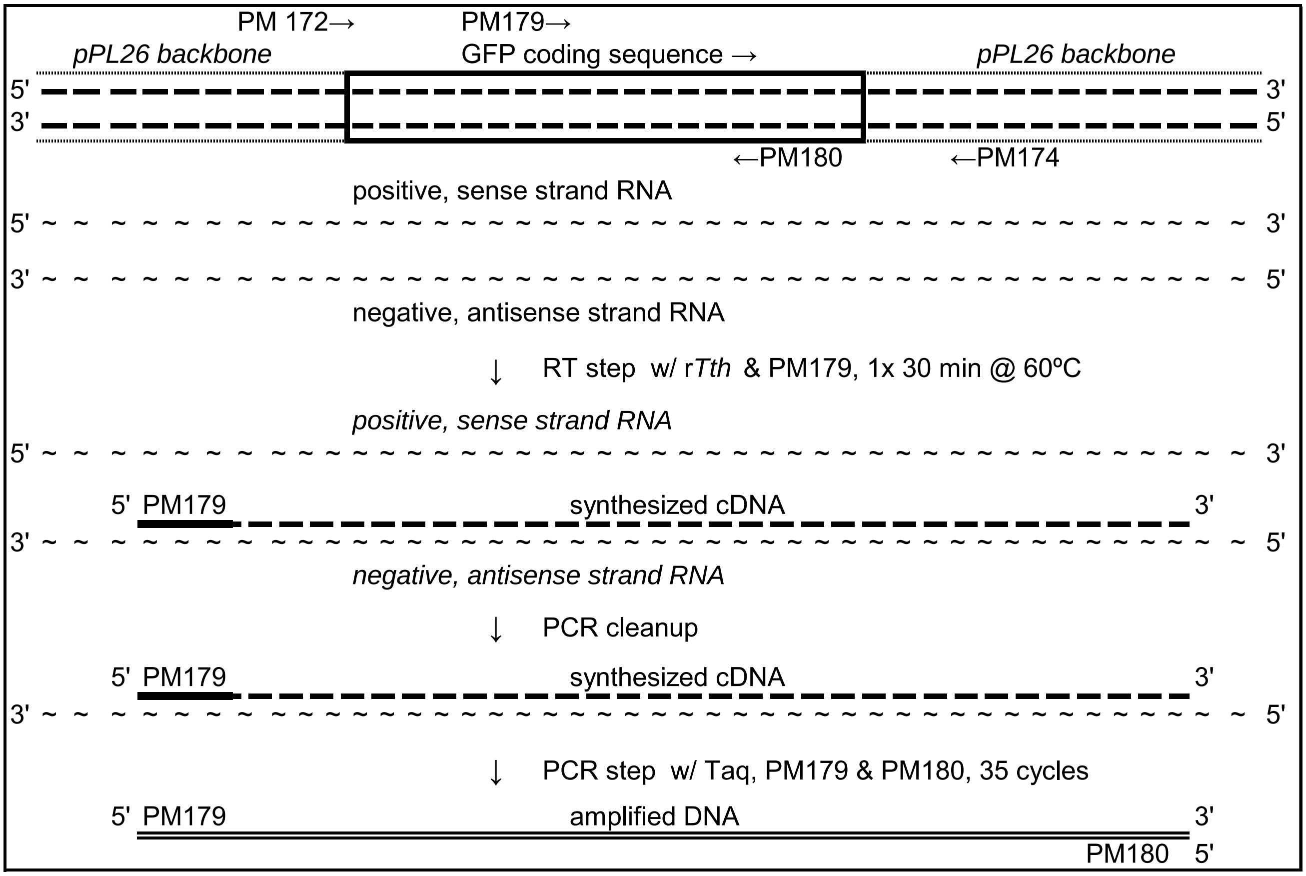

Figure 11: Strand-specific amplification with two step RT-PCR

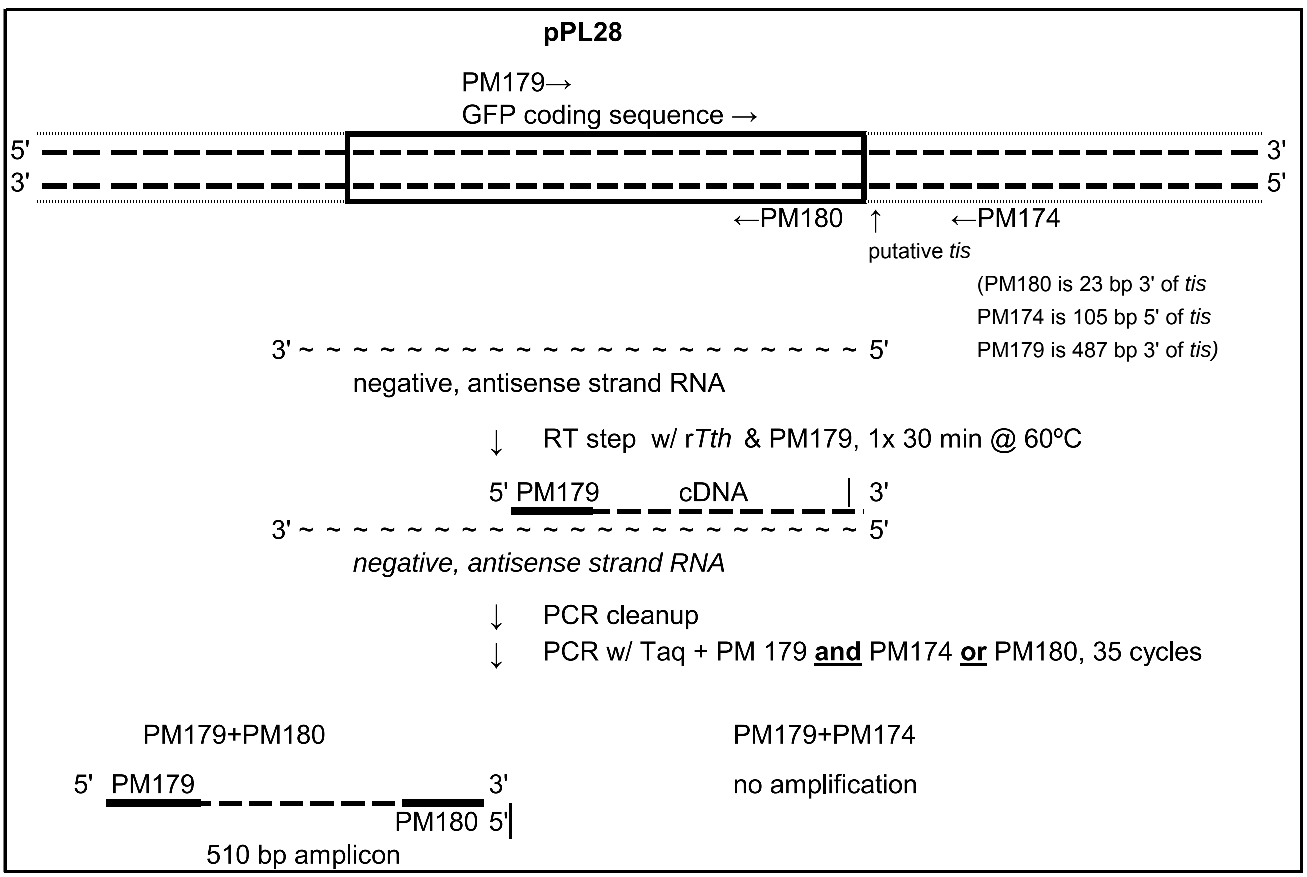

Figure 12: Delimiting *tis* location in pPL28 with two-step RT-PCR

the negative strand, which by definition is the transcription initiation site or *tis*. Using two different "antisense" primers, PM180 and PM174 in the PCR step, we got the expected, different results: PM179 + PM180 gave a 510 bp amplicon, while PM179 + PM174 gave no amplification, indicating that the *tis* site was upstream (5' on the negative strand) of PM180 and downstream (3' on the negative strand) of PM174. This limits the site of actual transcription initiation of the negative strand GFP RNA to approximately 23 bp downstream or 105 bp upstream of the putative *tis*, which suggests that the putative *tis* site identified by sequence homology is correct. Additional experiments to narrow this range with primers complimentary to sequences between PM180 and PM174 were not conclusive (the region is very AT rich, making good primer design difficult).

Plasmids pPL32 and pPL33, containing the complete ISAV segments 8 or 6, respectively, were constructed, as shown in Figure 13, from the backbone plasmid pPL26, as two of the final eight ISAV segment plasmids. For both plasmids, we started with RNA extracted from a known ISAV positive kidney sample. The ISAV segments were individually amplified by RT-PCR, using primers designed with their 3' ends complimentary to the published terminal sequences of ISAV segment 6 or segment 8 (Sandvik, 2000). In addition to their ISAV segment complimentary sequences, each of the primers had additional 5' sequences which contained a *BsmB*I restriction site. After amplification and gel purification, both ISAV segment cDNAs were ligated into a T/A pDrive cloning vector (Qiagen catalog # 231222), and transformed into *E. coli*. White transformant colonies were screened for inserts of the

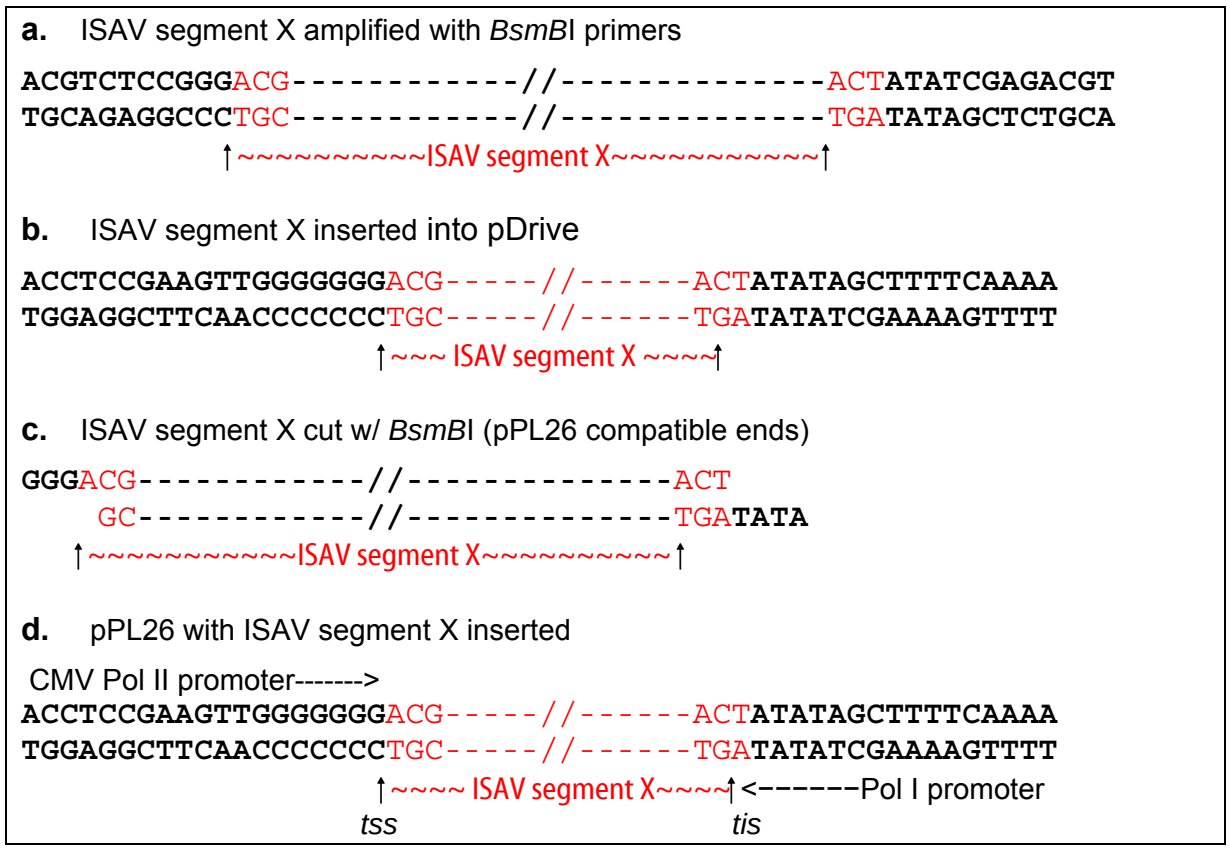

Figure 13: Cloning & Insertion of ISAV Segments into pPL26

correct size by PCR using primers complimentary to pDrive sequences on either side of the T/A insertion site. One transformant for each segment was grown up in culture and plasmid DNA purified by miniprep (Qiagen catalog # 27104). The plasmid minipreps were checked for correct insert size and orientation by restriction digests, then the ISAV segments were cut out of the pDriver vector using *BsmB*I, and gel purified. The purified fragments were ligated into *BsmB*I cut (linearized) plasmid pPL26, and transformed into *E coli*. Transformants were screened by PCR for correct insert size and orientation by restriction digestion.

We sequenced the ISAV segment inserts in plasmids pPL32 and pPL33 and, unfortunately, both contained several base changes from wild-type ISAV sequence, and appeared to be PCR-induced errors. We had used the single-step RT-PCR enzyme r*Tth* and segment 6 or segment 8 terminal primers to amplify these segments from an ISAV infected kidney RNA prep. In retrospect, this was probably not the correct choice of enzyme: r*Tth* functions as both a reverse transcriptase and a DNA polymerase in the presence of Mn^{2+} (instead of the Mg^{2+} typically used in PCR buffers); however the Cold Spring Harbor Cloning Manual (Sambrook & Russell, 2001) notes that the fidelity of

most DNA polymerases is lowered in the presence of manganese ions relative to their fidelity with magnesium ions. We subsequently switched all PCR or RT-PCR reactions where fidelity was critical to using Thermoscript (for RT) and Platinum Taq Polymerase, high fidelity (for PCR) (both enzymes from Invitrogen, catalog # 11146-057), and are currently repeating the amplification and cloning of segments 6 and 8 into the backbone expression plasmid (pPL26) using these enzymes. Although the internal sequences of plasmids pPL32 and pPL33 disappointingly had base changes, the ends of both inserts were correct, indicating the success of the cloning strategy we had designed for inserting the ISAV genomic segment sequences into our expression plasmid with no extraneous bases at either end (using the offset cutting restriction enzyme *Bsm*BI and primers containing *Bsm*BI recognition sites; see figure 5).

Given our frustration at our slow pace of determining both the exact 5' end sequences of ISAV segments 1-5 and 7, and the *Salmonid* Pol I promoter *tis* sequence, plasmid pPL34 was designed and constructed as a way to prove that the ISAV reverse genetics system and our plasmids would ultimately function as designed. Although demonstration of the production of negative strand GFP RNA from pPL28 transfected cells indicated that the cloned *Salmonid* Pol I promoter was functioning, it did not address whether such plasmid encoded negative strand RNA could or would be packaged in virions. Since there are no "helper viruses" known in the ISAV- salmonid cell system, unlike the situation with influenza virus and mammalian cells (Fodor, 1999, Schickli, 2001), packaging of plasmid-encoded RNAs into viral particles could only be demonstrated in ISAV infected cells – which in turn required that any plasmid-encoded RNA be distinguishable from the native ISAV segment RNAs. GFP RNA from plasmid pPL28 does not have the conserved, complimentary hairpin ISAV sequence ends, and therefore would not be expected to be packaged in viral particles. While ISAV segment 8 and segment 6 RNAs from plasmids pPL32 and pPL33 do have the hairpin end ISAV sequences crucial for packaging, they are not distinguishable from native ISAV segment 8 and segment 6. Fortuitously, the native ISAV segment 6 sequence contains two Bgl II restriction sites 59 bases apart, approximately in the middle of the segment (Figure 14), which would allow RNA molecules produced from a segment 6 plasmid with the sequence between the *Bgl* II sites deleted (segment 6Δ RNA) to be distinguished from native ISAV segment 6. Accordingly, we constructed plasmid pPL34 in the same manner as plasmid pPL33, except that the ISAV segment 6 plasmid in pDrive was cut with *Bgl* II and religated to delete a small 59 bp sequence of the native ISAV segment 6 before cloning into pPL26. We designed an "indel" primer, PM184, whose sequence spanned the deletion (Figure 14), and using pPL33 and pPL34 DNA determined optimal PCR conditions that allowed PM184 to amplify the deleted sequence (pPL34), but not the undeleted, native sequence (pPL33), thereby allowing PCR with PM184 to distinguish deleted and native ISAV segment 6. An additional assumption of this approach was that the plasmid-encoded segment 6Δ RNA would be packaged/copackaged in viral particles even in the presence of competing, native segment 6 RNA. This seemed a reasonable assumption for four reasons: (i) Plasmid RNA expression is driven by the constitutive *Salmonid* ribosomal RNA Pol I promoter and therefore would likely result in high intracellular levels of segment 6Δ RNA. (ii) There was no selective disadvantage for packaging of the segment 6Δ RNA, since there was no requirement for further infectivity. (iii) With PCR, even low levels of co-packaging could be detected. (iv) Copackaging has been observed in viral co-infection experiments in other systems (Bennett, 1999).

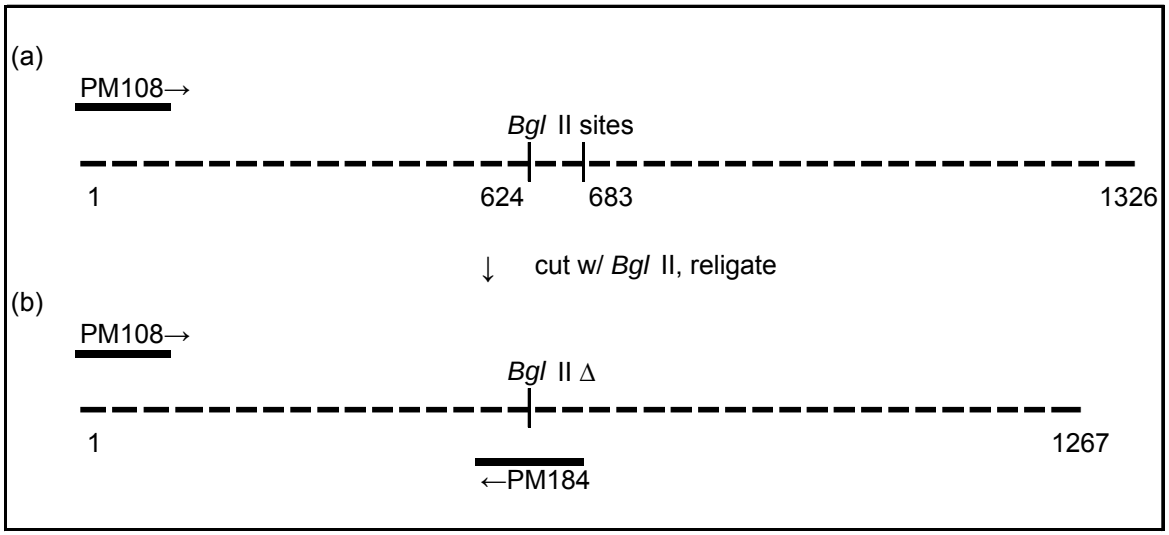

Figure 14: (a) cloned ISAV segment 6 with internal *Bgl* II sites. (b) cloned ISAV segment 6Δ with "indel" spanning primer PM184

Plasmid pPL34 DNA was transfected into CHSE cells, then after 6 days, the transfected cells were infected with native ISAV (confluent cells, 1000 viable ISAV particles per well). CPE was observed 14 days after ISAV inoculation. At 28 days after infection, culture supernatant and cells were separately harvested, and separate total

RNA preps made from each. Residual plasmid DNA, was eliminated from the RNA preps by treating them with DNase and confirmed by PCR, as described above for the GFP experiments. Conventional RT-PCR with ISAV segment 8 primers on the cell supernatant RNAs confirmed that the transfected/infected cells were producing extra-cellular viral particles. The supernatant RNA preps were treated with DNase and checked for the absence of plasmid DNA, in a similar manner to the GFP plasmid experiments described above. Testing these RNA preps by (conventional) RT-PCR with segment 6Δ primer PM184 and PM108 (Figure 14) gave the expected results: RNA preps from supernatants from transfected+infected cells were PCR positive, while RNA preps from supernatants from infected only cells (not transfected with pPL34) were PCR negative. These results demonstrate that negative strand segment 6Δ RNA is present in extra-cellular viral particles, which in turn indicate both that (i) the gene expression control signals in the dual functional expression plasmid pPL26 were functioning as designed; and (ii) that the ends of the plasmid-encoded ISAV genomic segment RNA are correct and sufficient to cause packaging of the RNAs into viral particles.

To summarize our significant accomplishments and findings during the period of this grant:

1. We identified errors or incomplete sequences in the published GenBank ISAV sequences for seven of the eight ISAV genomic segments. After trying a variety of methods over the course of the grant period, we believe we have finally developed a procedure that will allow determination of the correct nucleotide sequence of both the 3' and 5' ends of all eight ISAV genomic segments.

2. We optimized procedures for high efficiency plasmid transfection into salmonid cells Transfection rates of up to 45% were obtained using a CHSE cell line with the "Transit LT" transfection reagent (Mirus, catalog # MR2300) giving the best results.

3. We identified and cloned a *Salmonid* Pol I promoter. Using published primers in conserved regions of the ribosomal rRNA locus (Reed. 2000) we cloned and then subcloned a fragment from *Salmo salar* from the Intergenic Spacer region and identified by sequence homology a putative Pol I promoter region.

4 We constructed and tested a multifunctional expression plasmid backbone capable of promoting both mRNA and protein expression and negative strand viral (non-mRNA) molecules. We constructed a dual functional expression plasmid backbone for producing both positive strand mRNA and authentic, native negative strand viral RNA from the same ISAV segment insert. The CMV Pol II promoter and the SV40 late poly-adenylation signal are used to drive expression of mRNA. The cloned *Salmonid* Pol I promoter and a synthetic murine Pol I terminator drive expression of full-length uncapped, not-polyA -tailed viral RNA molecules. The remote cutting restriction enzyme *Bsm*BI and adapters containing *Bsm*BI recognition sites were used to allow cloning of all eight ISAV segment into the expression plasmid backbone in a manner that allows expression of native ISAV viral RNA segments with no extraneous or foreign bases at either end.

5. We determined the interferon production status of salmonid cell lines. Using detection of Mx protein, an interferon mediated anti-viral protein, we determined the interferon status of CHSE, ASK, and SHK. Mx protein was detected at low level in all cell lines.

6. We constructed and demonstrated functional protein expression from a plasmid transfected into salmonid cells. We constructed a plasmid with the GFP coding sequence inserted into our expression plasmid backbone, transfected this plasmid into a salmonid cell line and were able to detect both GFP mRNA and GFP protein.

7. We demonstrated functionality of the *Salmonid* Pol I promoter in salmonid cells. We used the same GFP plasmid transfected into salmonid cells described above to demonstrate, using strand-specific RT-PCR, the production of negative strand, anti-sense GFP RNA molecules demonstrating that our cloned Pol I promoter fragment was functional in salmonid cells.

8. We constructed ISAV transcription plasmids for two of the eight viral segments. We cloned the complete ISAV segment 6 and segment 8 genomic sequences into our backbone expression plasmid and were able to demonstrate production of both mRNA and viral RNA from the segment 6 plasmid. Although when we sequenced these plasmid inserts both exhibited several single base changes from the published ISAV genomic sequences - probably PCR induced mutations - both inserts had the expected and correct terminal sequences, demonstrating the success of our cloning strategy. Using a segment 6 deletion expression plasmid, we were able to demonstrate packaging of negative strand, viral RNA produced from this plasmid into viral particles.

B. Significant problems

As discussed at length above, the most significant and unexpected problem we faced carrying out this project was that of determining the correct terminal sequences of the ISAV genomic segments. We believe we have now devised a method for circumventing the technical problems of sequencing the end of RNA molecules and are currently continuing work to complete the ISAV segment end sequences. Another unexpected problem was the discovery that Pol I promoters, necessary for the production of non-mRNA viral RNAs, are extremely species specific in their activity, and no *Salmonid* Pol I promoter had been identified. We were able to successfully solve this problem by identifying and cloning a *Salmonid* Pol I promoter and show that this promoter sequence was functional in CHSE cells. One additional problem that we were not able to solve during the project period was finding an interferon negative salmonid cell line, which will be necessary for growth of attenuated NS-1del ISAV virions; however we recently received another CHSE cell line, CHSE-114, reported to be interferon negative (from John Kaufman, Oregon Department of Fish & Wildlife) and are currently confirming that it is IFN negative.

C. Need for additional work

Tasks that need additional work to allow completion of the ultimate project goal – production of complete, infectious, reverse-genetic engineered and attenuated ISAV virions – are as follows:

(i) complete determination of all ISAV segment 3' and 5' terminal sequences
(ii) construct 8 dual functional ISAV segment expression plasmids
(iii) construct ISAV segment 7 NS-1 gene deletion expression plasmids
(iv) find or make an interferon negative (IFN-) salmonid cell line
(v) transfect entire ISAV plasmid set and demonstrate virion production
(vi) test and demonstrate virulence (NS-1+) and attenuation (NS-1 del) on IFN+ and IFN- salmonid cell lines

VII. Project Evaluation

A. Extent to which the project goals and objectives were attained

Although the final project goal – production of complete, infectious, reverse-genetic engineered and attenuated ISAV virions – was not accomplished during the grant period, we believe the data from the experiments were able to complete give a strong indication of the ultimate success of the project. We have constructed and shown that the dual functional plasmids, containing ISAV genomic segments under the control of mRNA (Pol II, poly-a tail) and non-mRNA (Pol I and Pol I terminator) gene control signals do produce both positive and negative strand RNAs, and that a viral genomic negative strand transcribed from a plasmid can be packaged into extracellular viral particles. The individual specific tasks proposed in the original project plan, and whether they were accomplished or modified are shown in Table 3.

planned project experimental tasks		accomplished or modified?	explanation
1-1	Plan plasmid construction strategies	yes	
1-2	Construct GFP protein expression test plasmid	eliminated, unnecessary	generic GFP plasmid worked
1-3	Construct transcription test plasmid	eliminated; accomplished in 1-5	used ISAV transcription plasmid
1-4	Construct ISAV protein plasmids (NP, PA, PB1 & PB2)	eliminated	switched from 8 RNA + 4 protein expression plasmids to 8 dual-functional plasmids
1-5	Construct ISAV transcription plasmids (genome segments 1-6, 8)	partially completed	*Salmonid* Pol I promoter identified & cloned; dual-functional plasmid backbone assembled; segment 6 and segment 8 expression plasmids constructed, but with PCR errors
1-6	Construct ISAV segment 7 plasmids (NS-1 + and NS-1 del)	no	late stage task dependent on 1-5; no experimental work
1-7	Purify prep quantities of entire ISAV plasmid set	no	late stage task dependent on 1-5 & 1-6; no experimental work
2-1	Plan cell lines & transfection optimization strategies	yes	

2-2	Transfect GFP test plasmid & demonstrate protein expression	yes	generic GFP plasmid worked
2-3	Use GFP plasmid to optimize transfection efficiencies in cell lines	yes	generic GFP plasmid worked
2-4	Transfect transcription test plasmid and demonstrate RNA production	yes	demonstrated mRNA & protein expression with GFP dual functional plasmid
2-5	Transfect ISAV protein plasmids and demonstrate mRNA & protein expression	modified	
2-6	Transfect ISAV genome segment plasmids and demonstrate RNA production	partially completed	demonstrated with segment 6 dual functional expression plasmid
2-7	Transfect NS-1 deletion plasmids & characterize RNAs produced	no	late stage task dependent on 1-6; no experimental work
2-8	Transfect entire ISAV plasmid set and demonstrate virion production	partially completed	demonstrated packaging of segment 6 Δ plasmid produced vRNA into extracellular virions
2-9	Expand reconstituted virion populations (NS-1 + and NS-1 del) on monolayers	no	late stage task dependent on 2-8; no experimental work
2-10	Test and demonstrate virulence (NS-1+) and attenuation (NS-1 del) on IFN+ and IFN- cell lines	no	late stage task dependent on 2-9; no experimental work

Table 3 Project task accomplishments & modifications

B. Dissemination of Project Results

Results of this project have been submitted to NMFS and the S-K Program Office in the form of semi-annual progress reports and this final project report, and will also be published in peer-reviewed scientific journals such as 'Diseases of Aquatic Organisms' and 'Journal of Virology'. Corrected and complete ISAV segment sequences will be deposited in GenBank Scientific presentations will also be given at national and international aquaculture conferences.

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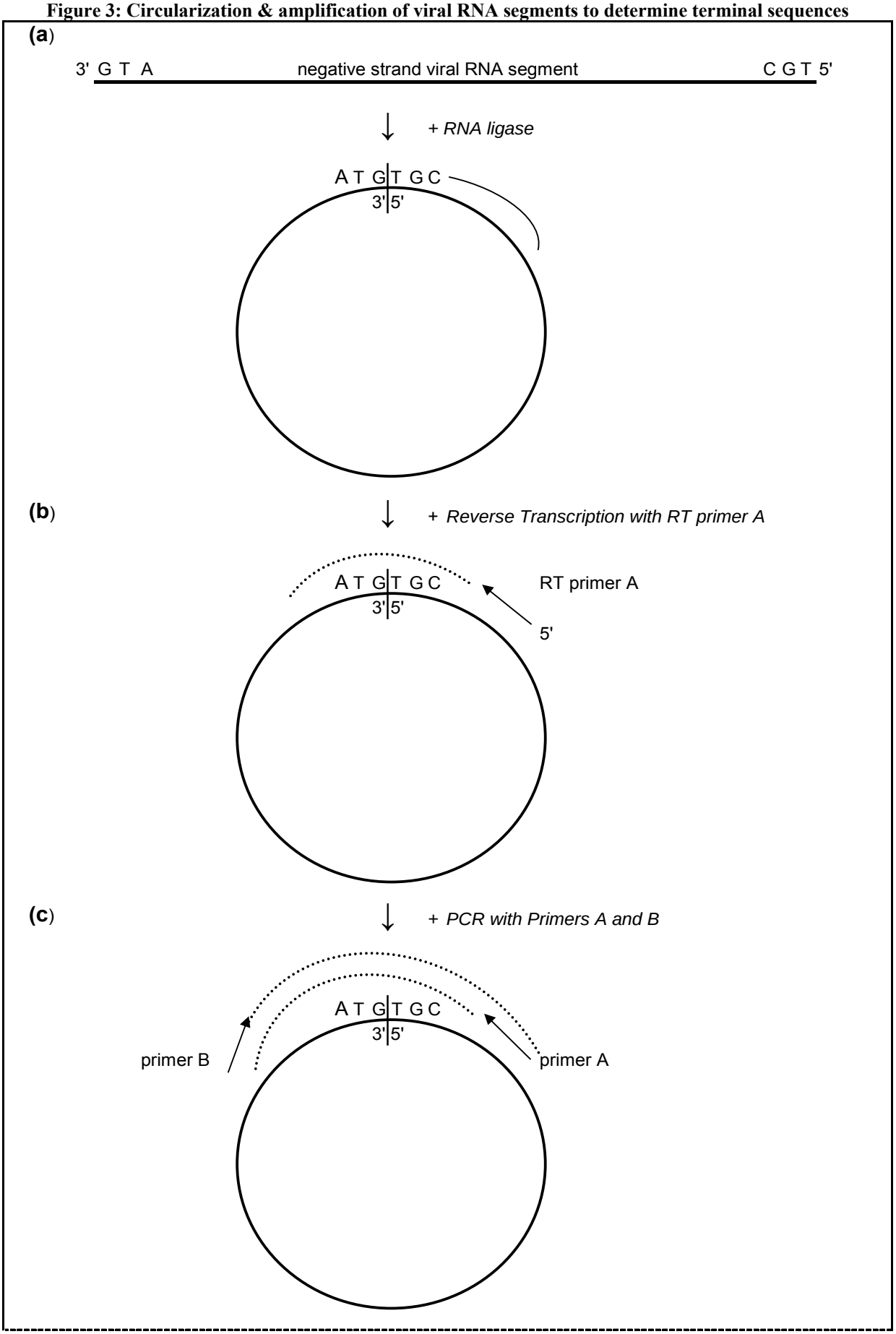
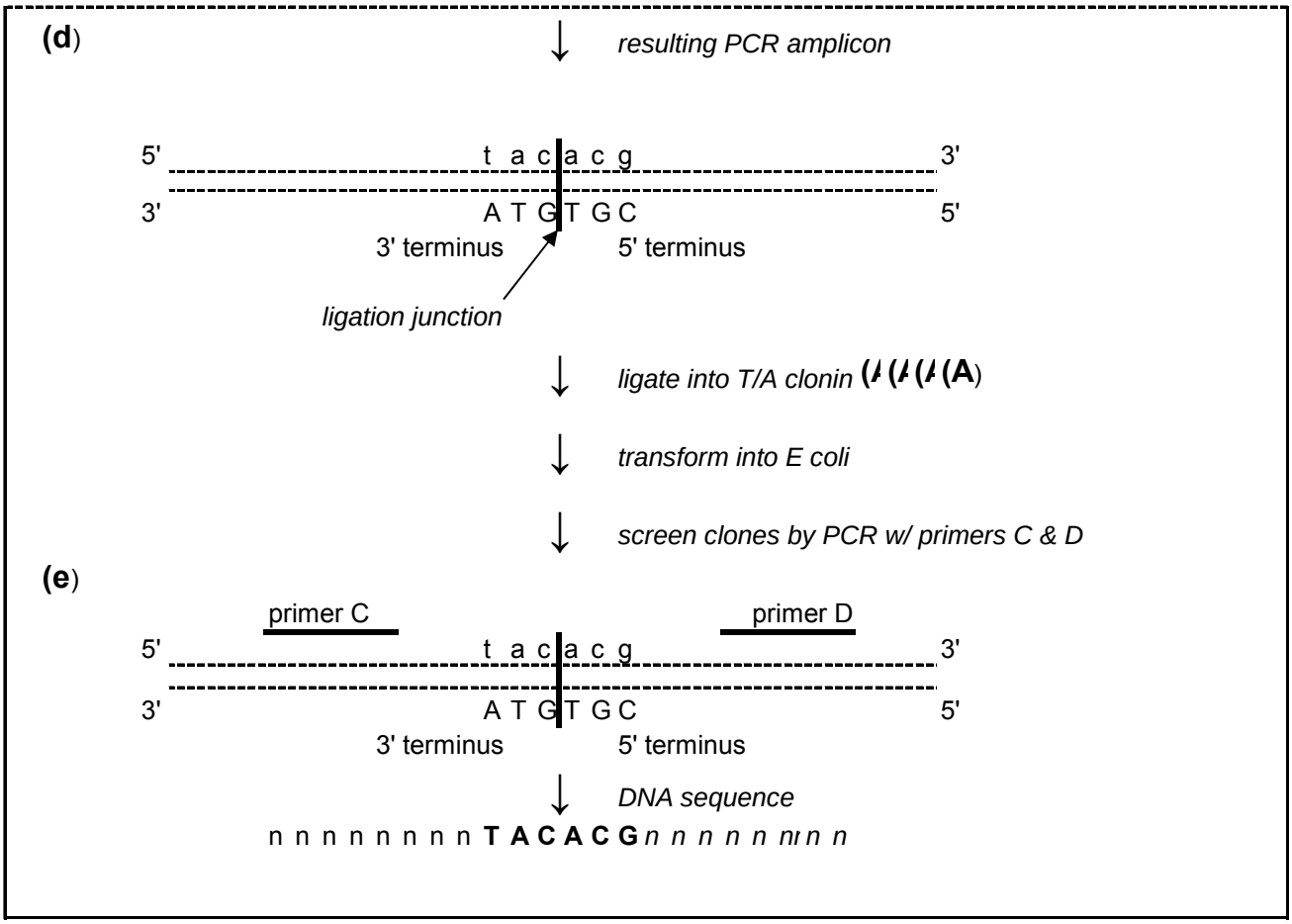
Figure 3: Circularization & amplification of viral RNA segments to determine terminal sequences




(d)
resulting PCR amplicon
5'
t a c a c g
3'
3'
A T G T G C
5'
3' terminus
5' terminus
ligation junction
ligate into T/A clonin (A (A (A (A)
transform into E coli
screen clones by PCR w/ primers C & D
(e)
primer C
primer D
5'
t a c a c g
3'
3'
A T G T G C
5'
3' terminus
5' terminus
DNA sequence
n n n n n n n n T A C A C G n n n n n n n n n